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Light regulation of vitamin C in tomato fruit is mediated through photosynthesis

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Abstract

Higher levels of irradiance result in higher accumulation of ascorbate in leaves and fruits. Photosynthesis and respiration are an integral part of the physiological mechanism of light regulation of ascorbate in leaves, but little is known about the light regulation of ascorbate in fruit. The aim of this study was to investigate whether fruit illumination alone is sufficient for ascorbate increase in tomato fruit and whether this light signal is mediated by respiration and photosynthesis. First the changes of ascorbate with the progress of fruit development were investigated and subsequently detached fruit of different tomato genotypes were exposed to different irradiances and spectra. Measurements were performed on ascorbate, respiration, photosynthesis and chlorophyll content of the fruit. When attached to the plant, there was no effect of development on ascorbate from the mature green to the red stage. Detached fruit stored in darkness did not accumulate ascorbate. However, when exposed to 300-600 µmol m^{-2} s^{-1} light detached mature green fruit (photosynthetically active) substantially accumulated ascorbate, while mature red fruit (non-photosynthetically active) did not respond to light. Photosynthesis correlated with this increase of ascorbate while no correlation between respiration and ascorbate was found. Spectral effects on ascorbate in detached tomato fruit were limited. These results indicate that the signal for light regulation of ascorbate is perceived locally in the fruit and that fruit illumination alone is sufficient for a considerable increase in ascorbate levels for as long as the fruit contains chlorophyll. It is shown that photosynthetic activity of the fruit is an integral part of the response of ascorbate to light in tomato fruit. The light induced increase in ascorbate levels occurred in a range of genotypes, indicating a universal effect of light to ascorbate in tomato fruit.

Keywords: vitamin C, ascorbic acid, irradiance, spectrum, photosynthesis, respiration

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1. Introduction

L-ascorbate (ASC; vitamin C; CID 54670067) is an antioxidant compound found in considerable amounts in plant tissue. In addition to its antioxidant activity, ASC has pro-oxidant effects. ASC is a compound with high potential for cancer prevention and treatment in humans (Chen et al., 2007, 2005; Du et al., 2012). ASC deficiency in the human body has been related to the coronary heart disease and diabetes mellitus (Boekholdt et al., 2006; Li and Schellhorn, 2007; Mandl et al., 2009; Paolisso et al., 1994). Due to its widely proclaimed beneficial effects on the human immune system, ASC has been characterized as essential for human health (EFSA Panel on Dietetic Products, 2010). The human organism through the progress of evolution lost the ability to synthesize ASC (Asensi-Fabado and Munné-Bosch, 2010; Chatterjee, 1973; Nishikimi et al., 1994). Plants are considered the most important source of ASC for humans as the bioavailability of ASC from plants is higher than that from artificial supplements (Bjelakovic et al., 2004; Fitzpatrick et al., 2012; Inoue et al., 2008).

One of the most widely discussed abiotic factors affecting ASC in plants is light. Increasing the irradiance level, increases ASC levels of leaves (Bartoli et al., 2006; Dowdle et al., 2007; Fukunaga et al., 2010). A response of ASC to light has also been reported for fruits like kiwi (Li et al., 2010), tomato (Massot et al., 2012; Ntagkas et al., 2016), apple (Li et al., 2009) and grapefruit (Cakmak et al., 1995). Shading of tomato fruit while still growing attached to the plant resulted in lower ASC levels (Gautier et al., 2008). When tomato fruit were illuminated with LEDs while still on the plant, they achieved higher ASC levels than in non-illuminated fruit (Labrie and Verkerke, 2012). Therefore, it is possible that the light signal for increase of ASC levels of the fruit is perceived locally from the fruit.

The spectrum of light may also affect ASC in plants. Increasing the red:far red ratio increased the ASC concentration in bean seedlings (Bartoli et al., 2009). Blue light also increased ASC levels in lettuce, spinach and other leafy vegetables (Lester, 2006; Ohashi-Kaneko et al., 2007). The situation seems to be complicated as UV light enhanced ASC levels in soybean seedlings (Xu et al., 2005) while UV-B and UV-C light reduced ASC in tomato fruit (Giuntini et al., 2005; Maharaj et al., 2014).

The photosynthetic electron transport rate regulates gene expression of ascorbate peroxidase which affects the ability of the tissue to scavenge H_2O_2. This signal is mediated via changes in the redox state of the plastoquinone pool (Karpinski et al., 1997). In arabidopsis leaves, photosynthetic inhibitors such as DCMU and ATZ, arrested the accumulation of ASC under light, by reducing the activity of the D-Man/L-Gal biosynthetic pathway (Yabuta et al., 2007). Inhibition of photosynthesis in green tomato fruit also resulted in arrested ASC levels in the fruit (Badejo et al., 2012). The rate of photosynthetic electron transport is also linked to ASC turnover with limited effects however on ASC levels (Karpinski et al., 1997).

Respiration is another physiological process mediating light regulation of ASC. The last enzyme in the D-Man/L-Gal biosynthetic pathway (GLDH) is located in the inner membrane of mitochondria (Bartoli et al., 2000). GLDH is a functional part of complex I of the respiratory electron transport chain (Schimmeyer et al., 2016) and an electron donor to cytochrome C. Oxidized cytochrome C results in higher ASC levels as proven in isolated potato mitochondria (Bartoli et al., 2000). Inhibition of respiration at the level of complex I

(via application of KCN and rotenone) result in a considerable reduction in ASC levels (Bartoli et al., 2006; Millar et al., 2003). The alternative oxidase (AOX) respiratory pathway has also been associated to ASC biosynthesis. ASC accumulated in leaves of AOX overexpressing arabidopsis mutants while it did not in the wild type (Bartoli et al., 2006). It remains elusive whether respiration and photosynthesis mediate the signal for increase of ASC when tomato fruit are treated with higher irradiances. Respiration and photosynthesis possibly interact in light regulation of ASC (Ntagkas et al., 2017).

It is known that light regulates ASC levels in leaves and that respiration and photosynthesis are an integral part of this regulatory mechanism. The aim of the present study was to investigate whether the signal of light for increase of ASC levels in tomato fruit is perceived locally in the fruit and whether this light signal is mediated by respiration and photosynthesis. It was hypothesized that light may increase both respiratory and photosynthetic activities and hence may stimulate ASC accumulation. To test these hypotheses first the changes of ASC with the progress of development were investigated and subsequently detached tomato fruit of different genotypes were exposed to different irradiances and spectra.

2. Materials and Methods

2.1 Plant material

Tomato fruit (*Solanum lycopersicum*) were harvested from the glasshouse of a commercial grower (Royal Pride Holland) in Middenmeer (N 52° 46' 58'', E5° 03' 42''), the Netherlands. Fruit were transported to Wageningen University and Research facilities in Wageningen, the Netherlands. In all experiments fruit were selected from the 3rd and 4th positions of the truss (counting acropetally) when they were within a specific range of colour, firmness and weight (Table 1). Only trusses at the same position on the plant with 8 fruit per truss were used. The developmental stage of the fruit was characterised based on lycopene (NAI) and chlorophyll (NDVI) content related indices (table 1). Five experiments were performed. In Experiments (Exp.) 1, 2, 3 and 5 the commercial cultivar Vimoso (40 g/fruit) was used. In Exp. 4, five commercial cultivars bearing mature fruit of different size (20 to 160 g/fruit; Table 1) were tested. For experiments 2 to 5 the light treatments begun approximately 5 hours after harvest. As difference experiments took place in different parts of the year, fruit for all experiments were picked from a greenhouse equipped with artificial light. This way the difference in growth irradiance was minimized to the best possible extend so that the fruit would have comparable initial ASC levels.

2.2 Set-up of experiments

In Exp. 1 the ASC levels of fruit of different developmental stages that matured in the greenhouse was measured. Four developmental stages were selected (mature green, breaker, red, advanced red). The fruit matured to the designated developmental stage while attached on the plant. The ambient irradiance in the greenhouse at fruit level was measured with a handheld quantum sensor (LI-250; Li-Cor Inc., Lincoln, Nebraska, USA). The measurement took place at noon with clear sky where the irradiance is expected to be at its highest. Irradiance was measured at fruit level at a total of 50 points of the row the fruit were harvested from. Irradiance at fruit level on June 16th (harvest point of Exp. 1) was found to be 98 $\pm$6.8 µmol m^{-2} s^{-1}.

In Exp. 2, in order to characterise ASC levels during ripening of detached fruit in light and darkness, mature green tomato fruit were placed for 15 days in 500 µmol m^{-2} s^{-1} of white light and darkness. In Exp. 3, the response of ASC levels to irradiance was studied by keeping mature green and red tomato fruit under a range of irradiance levels (0, 8, 144, 306 and 616 µmol m^{-2} s^{-1}) for 7 days. In Exp. 4, four tomato cultivars with different fruit size were exposed to 300 µmol m^{-2} s^{-1}, an irradiance level causing considerable accumulation of ASC (Exp. 3). In Exp. 2, 3 and 4 a broad spectrum (white light) was used to avoid lack of spectra potentially essential for increasing ASC. In Exp. 5 the effect of spectrum on ASC was tested. Tomatoes were placed at a combination of 100 µmol m^{-2} s^{-1} white background irradiance supplemented with 250 µmol m^{-2} s^{-1} of monochromatic light (red, blue and far-red). The green light treatment was an exception due to the output limitations of the LEDs. It was a combination of 150 µmol m^{-2} s^{-1} green light and 200 µmol m^{-2} s^{-1} of white light, such that total irradiance was kept constant.

In Exp. 2 to 5 after the transfer from the greenhouse, fruit were placed in a climate-controlled room which contained 5 compartments with one light treatment per compartment. The calyx was removed and the fruit were placed with the calyx scar pointing downwards to the table in order to minimize water loss. Fresh weight (FW) measurements

confirmed uniform water loss for all treatments. In Exp. 3, 4 and 5 fruit lost 0.62% of the initial weight after 7 days. In Exp. 2 fruit lost 2.4% of their initial weight after 15 days in the treatments. LED light was applied continuously (24 hours per day) in all experiments. The broad/white spectrum was supplied by blue phosphorous coated LEDs (GreenPower LED, Philips, The Netherlands). Far red was supplied by LED production modules (Green Power LED, Philips, The Netherlands). Blue and red light was supplied by LEDs with dominant wavelengths of 450 nm and 638 nm, respectively (types Royal Blue and Red Luxeon K2, Lumileds Lighting Company, San Jose, CA, USA). Green was supplied by custom made LED modules with dominant wavelength at 520 nm. The LEDs were suspended 80 cm from the surface of the bench. The sides and bottom of the compartments were covered with neutrally reflective MC-PET sheets (SRF-A032T, Sekisui Plastics Co., LTD, Osaka, Japan) in order to improve irradiance levels and light distribution. These reflective properties were verified with a spectrophotometer (USB-4000, Ocean Optics, Dunedin, FL, USA) with the use of an integrated sphere in an obscure box. Spatial distribution of irradiance and spectrum was measured in a 5 by 5 cm grid with a spectroradiometer (USB2000, Ocean Optics, Duiven, The Netherlands; calibrated against a standard light source).

The total illuminated area was 80 x 50 cm out of which an area of 60 x 30 cm was selected for the placement of samples based on the light distribution measurements. Light distribution within the selected area was within 10 µmol m^{-2} s^{-1} for all light treatments of all experiments and 0.5 µmol m^{-2} s^{-1} for the 8 µmol m^{-2} s^{-1} irradiance treatment of Exp. 3. To further ensure uniform exposure of all fruit to the light treatments, fruit were rotated daily within the selected area. Phytochrome stationary state (PSS) was calculated according to equation 1 (Sager et al., 1988).

$$PSS = (\sum_{300}^{800} N_\lambda \sigma_{r_\lambda})/(\sum_{300}^{800} N_\lambda \sigma_{r_\lambda} + \sum_{300}^{800} N_\lambda \sigma_{fr_\lambda}) \quad \text{(eq.1)}$$

Where N_λ: photon flux at wavelength λ (nm), $\sigma_{r\lambda}$: photochemical cross-section of red absorbing phytochrome state, $\sigma_{fr\lambda}$: photochemical cross-section of far-red absorbing phytochrome state. Air temperature was 18 ^oC, relative humidity was 70% and CO_2 concentration was between 352ppm and 428 ppm for all experiments. Fruit temperature was monitored with k-type thermocouples attached to the lower side of the fruit on TC-08 data loggers (Picotechnology LTD., Cambridge, UK; table 1). Thermocouples were calibrated in distilled water at freezing and boiling point. Fans were placed in the openings below and above the reflective material on the sides, to avoid temperature deviations. Eventually, the fruit temperature was approximately 0.5 ^oC higher than the air temperature with maximum temperature difference between the treatments of 0.4 ^oC (table 1).

2.3 Fruit colour and firmness

Changes in fruit colour were measured by a hand-held photodiode array spectroradiometer (PA1101, CP, Germany). Measurements were taken at three spots on the equatorial region of the fruit. This spectroradiometer provides the normalized anthocyanin index (NAI) calculated according to remittance spectra at 570 and 780 nm (R_{570} and R_{780} respectively) and the normalized difference vegetation index (NDVI) calculated according to remittance spectra at 660 and 780 nm (R_{660} and R_{780} respectively; Equations 2 and 3). NAI and NDVI values correlate with lycopene and chlorophyll contents respectively (Kuckenberg et al., 2008).

$$NAI = \frac{R_{780} - R_{570}}{R_{780} + R_{570}} \quad \text{(eq.2)}$$

$$NDVI = \frac{R_{780} - R_{660}}{R_{780} + R_{660}} \quad \text{(eq.3)}$$

Firmness of the fruit was measured by an acoustic detector (AFS, AWETA, Nootdorp, The Netherlands). The system measures the fruit weight (*m*, g/fruit) and the resonant frequency (*f*, Hz) of the fruit after it is hit by a small plastic piston. The firmness index (*Fi*) is calculated according to equation 4.

$$Fi = \frac{f^2 m^{2/3}}{10^6} \quad \text{(eq.4)}$$

2.4 Ascorbate and dehydroascorbate

L-ascorbate (ASC) and dehydroascorbate (DHA) were measured at the beginning of the treatments and after 7 days in Exp. 3, 4 and 5 or every 3 days in Exp. 2. For each treatment 10 fruit were combined in pairs into 5 replicates. Each replicate consisted of a pool of 6 pericarp discs of 1 cm diameter from two fruit. In tomato fruit ASC is mostly localized in the pericarp (Badejo et al., 2012). The discs were taken from the fruit side which was directly illuminated by the LEDs. The pericarp discs were then frozen in liquid nitrogen and grinded to fine powder.

0.2 g of fresh frozen tissue was thawed on ice in dark with the addition of 0.5 ml ice cold 3.3% meta-phosphoric acid. Samples were placed in an ultrasonic bath for 10 minutes and consecutively centrifuged at 25000 rcf at 4^{o}C for 10 minutes. 100µl of each sample was transferred to another HPLC vial where DHA was reduced to L-ASC with 50µl DTT 5mM and 400mM Tris base (Davey et al., 2003). The reduction of L-ASC to DHA took place in darkness for 15 minutes and was stopped with the addition of 50µl o-phosphoric acid 8.5%. Extracts were measured in a high-performance liquid chromatography system (ICS-5000, Dionex Corporation, Sunnyvale, USA). Calibration of the HPLC was performed with authentic ASC solutions of known concentration.

2.5 Measurements of respiration and photosynthesis

Respiration of individual tomato fruit was measured with a portable infra-red gas exchange system (LI-6400; Li-Cor Inc., Lincoln, Nebraska, USA). A single tomato fruit was placed in a transparent, hollow PVC sphere which was integrated at the sampling circuit of the LI-6400 with the cuvette bypassed (Savvides et al., 2013). The sphere reduced irradiance by 10% without affecting the spectrum. Gas exchange rates were measured both under light and darkness (dark respiration) by covering the sphere with a non-transparent hood. For fruit in darkness, the CO_2 rate was logged for 15 minutes after a stabilization period of 15 minutes. A chlorophyll fluorescence imaging system (FluorCam 700MF, Photon System Instruments, Brno, Czech Republic) was used to measure photosynthetic electron transport efficiency in photosystem II (ΦPSII) under 300 µmol m^{-2} s^{-1} with a saturating pulse of 3500 µmol m^{-2} s^{-1}. Maximum quantum efficiency of photosystem II (Fv/Fm) was measured after 30 minutes dark adaptation. Fv/Fm was measured under a saturating pulse of 3000 µmol m^{-2} s^{-1}.

FluorCam v.5.0 software was used to operate the measurement protocol in FluorCam 700MF.

2.6 Statistical analysis

One-way ANOVA was used to test the effects of the developmental stage on L-ASC (Exp. 1). Two-way analysis of variance (ANOVA) was used to test the effects of two factors on L-ASC (light treatment and time in Exp. 2, light treatments and developmental stage in Exp. 3, light treatments and cultivar in Exp. 4 and spectrum and time in Exp. 5). Individual plants were treated as independent replicates. This may have underestimated the random variance hence, we conducted our tests at $P=0.01$ instead of the commonly used $P=0.05$ with post hoc Tukey's honestly significant difference (HSD) multiple comparison tests ($P \leq 0.01$). Statistical analyses were carried out with the R software (R 3.0.1; R Project for Statistical Computing, Vienna, Austria).

Table 1: Overview of set-up of experiments investigating the effects of light on L-ASC in detached tomato fruit. Irradiance and PSS measurements represent the mean of 40 measurements equally distributed over the illuminated area. Data on fruit weight, NAI (lycopene index) and NDVI (chlorophyll index) are based on 10 replicate fruit and fruit temperature is based on 8 replicate fruit.

Experiment	Scope	Treatment Duration (days)	Spectrum	Irradiance (µmol m^{-2} s^{-1})	PSS	Tomato Cultivar	Average Fruit Temperature (°C)	Fruit Developmental Stage at Harvest	Average NAI	Average NDVI	Average Fresh Weight (g)
1	ASC time course of fruit on plant	-	-	-	-	Vimoso	-	Green Mature	-0.6 ±0.02	0.07 ±0.02	43 ±3
								Breaker	-0.3 ±0.04	-0.2 ±0.05	
								Red	0.2 ±0.01	-0.6 ±0.02	
								Advanced Red	0.6 ±0.01	-0.6 ±0.01	
2	Time course in light and darkness	15	Darkness	0	-	Vimoso	18 ±0.20	Green mature	-0.6 ±0.01	0.07 ±0.02	43 ±4
			White	500 ±10	0.83		18.4 ±0.13				
3	Response to irradiance	7	Darkness	0	-	Vimoso	18.1 ±0.12	Green mature	-0.6 ±0.02	0.07 ±0.01	43 ±3
			White	8 ±0.1	0.83		18 ±0.18	Red	0.2 ±0.02	-0.6 ±0.02	
			White	144 ±2	0.83		18.3 ±0.21				
			White	306 ±5	0.83		18.4 ±0.17				
			White	616 ±12	0.83		18.5 ±0.17				
4	Cultivar differences	7	Darkness	0	-	Robinio	18.4 ±0.13	Green mature	-0.6 ±0.01	0.07 ±0.02	21 ±2
			White	306 ±12	0.83	Vimoso					43 ±2
						Axiradius					101 ±11
						Roterno					98 ±9
						Komeet					160 ±17
5	Response to light spectrum	7	Red and white	253 and 122 respectively (375 ±10)	0.88	Vimoso	18.2 ±0.12	Green mature	-0.6 ±0.03	0.07 ±0.02	43 ±3
			Blue and white	255 and 118 respectively (373 ±11)	0.7		18.5 ±0.18				
			Far Red and white	258 and 119 respectively (377 ±12)	0.67		18.4 ±0.18				
			Green and white	155 and 209 respectively (364 ±12)	0.83						
			White	378 ±9	0.83		18.3 ±0.22				
			Darkness	0	-		18.1 ±0.17				

3. Results

3.1 ASC in tomatoes of different developmental stages

Tomato fruit of four different developmental stages were harvested and their ASC content was analysed (Exp. 1). During fruit maturation on the plant from mature green, breaker to red fruit lycopene index increased and chlorophyll index decreased, but ASC did not significantly change (Fig. 1). Red is the stage that fruit are typically harvested and extreme red refers to over-ripe fruit. In extreme red fruit ASC was 5-7 mg/100 FW higher than in the other developmental stages (Fig. 1).

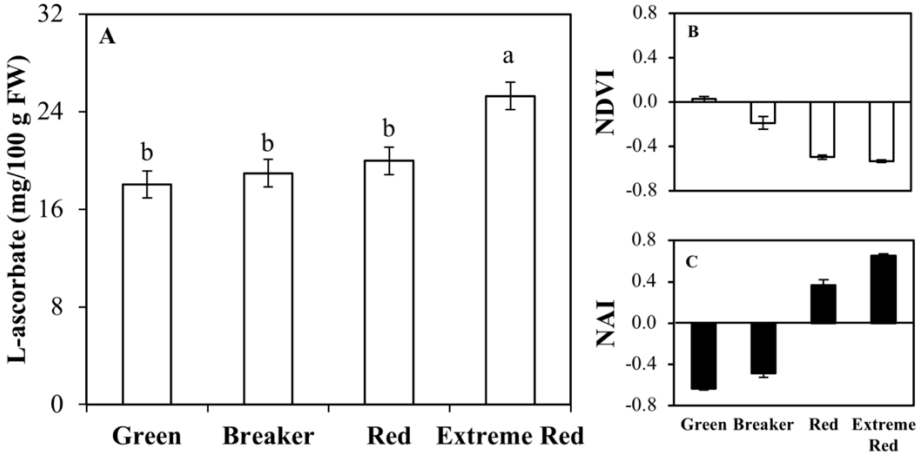

Figure 1: L-ascorbate concentration (A), chlorophyll index (NDVI, B) and lycopene index (NAI, C) of greenhouse grown tomato fruit harvested at 4 different developmental stages. Measurements were performed immediately upon harvest. Error bars represent standard errors of mean, letters indicate statistical difference at $P \leqslant 0.01$ with n=5 (Exp. 1).

3.2 Effects of light on ASC during ripening

Detached mature green tomato fruit were placed under white LED (500 µmol m^{-2} s^{-1}) light and darkness for 15 days (Exp. 2). During fruit ripening in darkness for 15 days ASC did not show any significant changes (Fig. 2). During fruit ripening under white light, ASC levels in the pericarp were increased 4.8 times from day 0 (mature green) compared to day 9 (breaker stage) with no further change thereafter. By the end of the experiment the fruit of both treatments had ripened to the red stage as indicated by the similar NAI and NDVI values (Fig. 2).

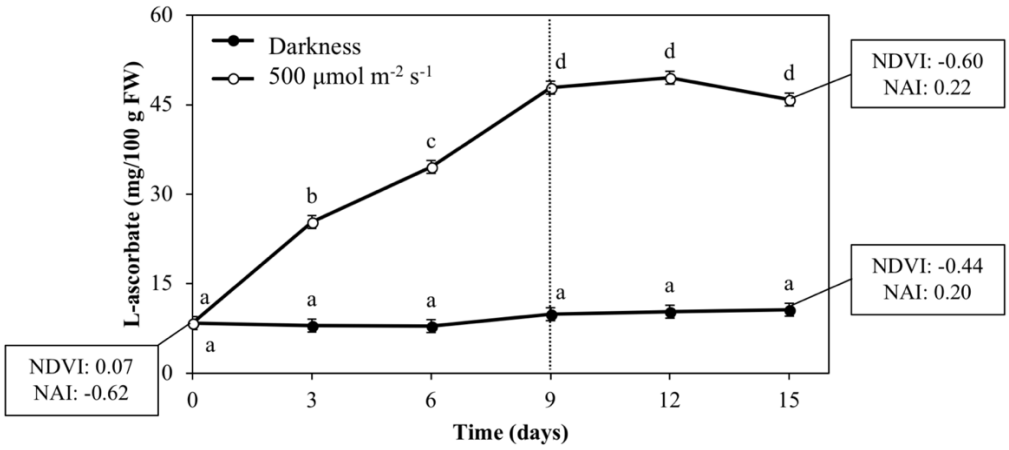

Figure 2: L-ascorbate concentration of tomato fruit kept under 500 µmol m^{-2} s^{-1} of white light (open symbols) and darkness (closed symbols) for 15 days. At the beginning of the treatments the fruit were in mature green stage. Broken line indicates the time point fruit entered the breaker stage. NAI and NDVI indices are presented for the initial and final time points. Error bars (when larger than symbol size) represent standard errors of mean, letters indicate statistical difference at P≤0.01 with n=5 (Exp. 2).

Efficiency of photosystem II (ΦPSII), maximum photosynthetic efficiency of photosystem II (Fv/Fm) and respiration were measured at day 2 and day 14 after treatments with white LED light (500 µmol m^{-2} s^{-1}) and in darkness (Exp. 2). ΦPSII was found to be significantly higher in fruit that were kept in light (500 µmol m^{-2} s^{-1}) compared to fruit in darkness (2 and 14 days; Fig. 3A). Fv/Fm was not different between fruit held in light and darkness at 2 days. At day 14 however, fruit in the light treatment had significantly lower Fv/Fm in comparison to fruit in the dark treatment (Fig. 3B). The light treatment might have a small effect on ripening as NDVI was slightly lower in the light treatment that results in the observed reduction in the Fv/Fm. However, this is most likely within a range that is not expected to affect ASC levels as observed in Exp. 1.

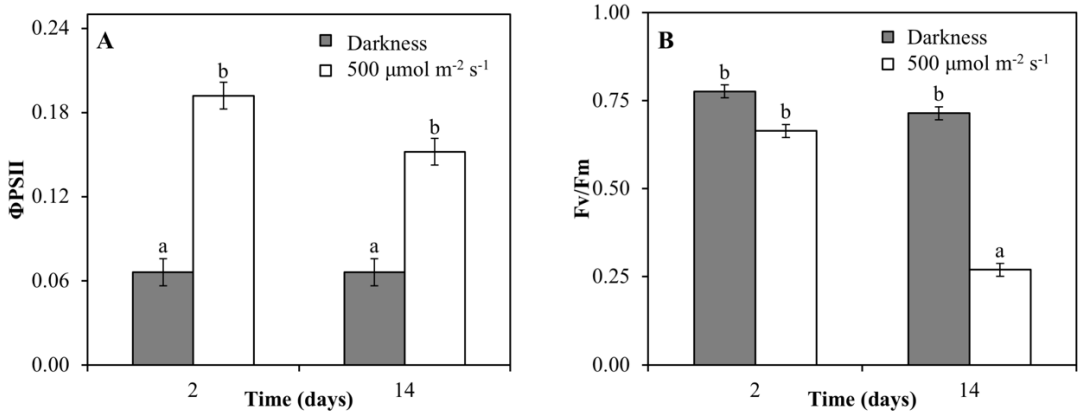

Figure 3: (A) Efficiency of photosystem II (ΦPSII) and (B) maximum photosynthetic efficiency of photosystem II of tomato fruit kept under 500 µmol m^{-2} s^{-1} (white bars) and darkness (black bars). Measurements took place after 2 days and after 14 days in the treatment. Error bars represent standard errors of mean, letters indicate statistical difference at P≤0.01 with n=5 (Exp. 2).

At the second day of the treatment dark respiration rates were significantly lower in the light treatment compared to the dark treatment, while at day 14 there were no significant differences anymore in dark respiration (Fig.4). There was no difference between light and dark respiration in the light treatment (Fig.4).

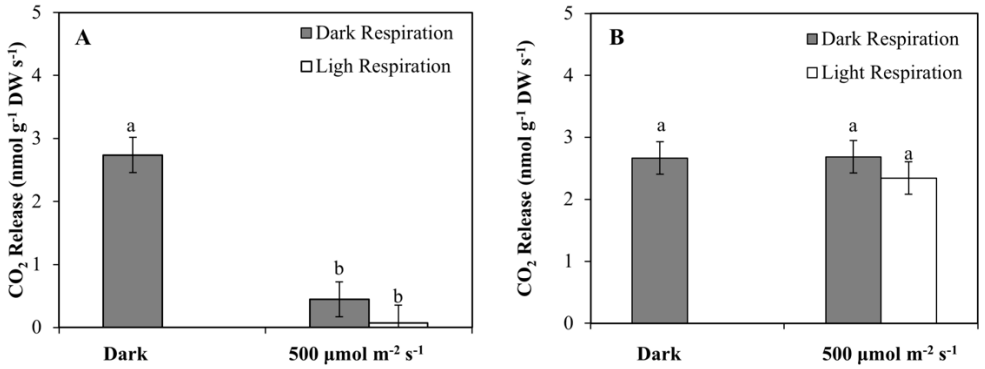

Figure 4: Rate of CO_2 release from tomato fruit kept in darkness or 500 µmol m^{-2} s^{-1} white light. Measurements took place at day 2 (A) and day 14 (B) of the treatment. Dark bars indicate respiration in darkness (CO_2 release in darkness) while white bars represent respiration in light (CO_2 release in light). Respiration under light was not measured for fruit kept in dark. Error bars represent standard errors of mean, letters indicate statistical difference at P≤0.01 with n=5 (Exp. 2).

3.3 Effects of irradiance on ASC in green and red fruit

At start the ASC levels were similar in red and green fruit (Fig. 5). Applying different irradiances in the range of 0 to 600 µmol m^{-2} s^{-1} to mature green fruit, showed that the treatment of 300 µmol m^{-2} s^{-1} yielded the highest observed ASC levels. Irradiances below 150 µmol m^{-2} s^{-1} did not significantly affect the ASC concentration. The ASC concentration in red fruit was not significantly affected by irradiance. Initial ASC levels of green tomatoes did not differ significantly from that of red tomatoes (15 and 17 mg 100 g^{-1} of fresh weight, respectively).

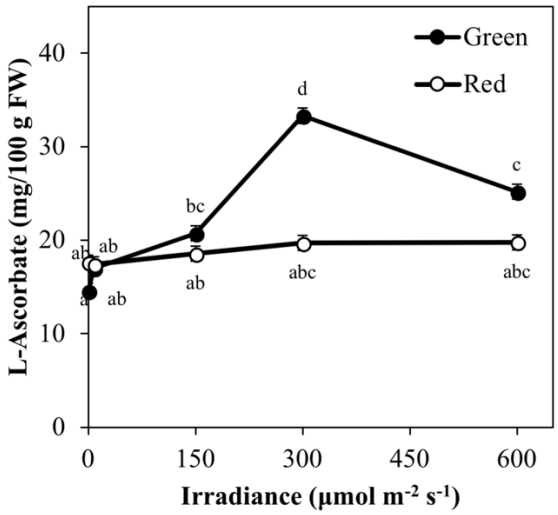

Figure 5: Effect of irradiance on L-ascorbate concentration in tomato fruit after 7 days light treatment on detached fruit. At the beginning of the experiment (t=0) mature green and red fruit were picked from the plant and irradiance treatments were applied. Error bars represent standard errors of mean, letters indicate statistical difference at P≤0.01 with n=5 (Exp. 3).

3.4 ASC increases with higher irradiance in several tomato cultivars

The effect of 7 days of white LED light (300 µmol m^{-2} s^{-1}) on ASC was investigated in detached mature green fruit from five different cultivars grown under comparable conditions in a commercial greenhouse (Exp. 4). In darkness ASC levels did not increase in any of the cultivars (Fig. 6). In the light, ASC levels of all five tomato cultivars increased during ripening of mature green fruit compared to fruit that ripened in darkness (Fig. 6). Depending on the cultivar the increase varied from 1.8 to 2.4 times (Fig. 6).

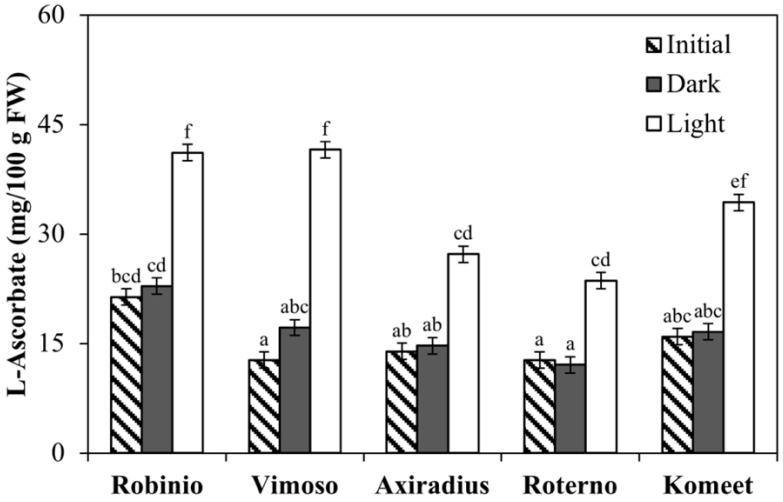

Figure 6: L-ascorbate concentration of fruit of five commercial cultivars (robinio, vimoso, axiradius, roterno and komeet) kept under 300 µmol m^{-2} s^{-1} white light (white bars) and darkness (black bars) for 7 days. Fruit from all cultivars at the beginning of the treatment (green bars) were at the mature green stage. Error bars represent standard errors of mean, letters indicate statistical difference at P≤0.01 with n=5 (Exp. 4).

3.5 Spectral effects on ASC

To study the effect of spectrum on ASC levels (Exp. 5) detached tomato fruit were kept for 7 days under background white LED light (100 µmol m^{-2} s^{-1}) while supplemented with monochromatic LED light (250 µmol m^{-2} s^{-1}). ASC levels in all light treatments were significantly higher compared to the darkness and the initial ASC levels. The highest levels of ASC were achieved in the blue treatment (Fig. 7), which was about 10% higher than fruit under white light. The ASC level of fruit under red and green light did not significantly differ from those under white light (Fig. 7). Far-red resulted in significantly lower ASC levels compared to all other light treatments (Fig. 7).

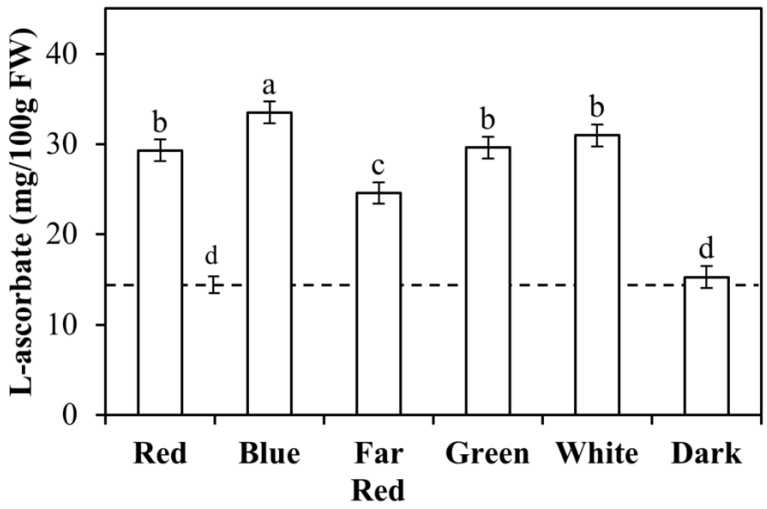

Figure 7: Effect of light spectrum on L-ascorbate concentration of tomato fruit. Detached fruit were kept for 7 days under 250 µmol m^{-2} s^{-1} of monochromatic (red, blue and far-red) light combined with 100 µmol m^{-2} s^{-1} of white light; total irradiance was 350 µmol m^{-2} s^{-1} in all treatments. However, the green light treatment was a combination of 150 µmol m^{-2} s^{-1} green light and 200 µmol m^{-2} s^{-1} of white light. There was also a darkness treatment. Broken line indicates L-ascorbate levels at the beginning of the light treatments (t=0). All light treatments had the same light sum. Error bars represent standard errors of mean, letters indicate statistical difference at P≤0.01 with n=5 (Exp. 5).

4. Discussion

4.1 ASC in tomato fruit does not increase substantially with the progress of development

For detached tomato fruit, ASC does not increase considerably with the progress of development (from mature green to red) under irradiances below 150 µmol m^{-2} s^{-1}. This is in line with the absence of developmental effects on ASC content of tomato fruit when tomatoes grown in a greenhouse at the lower part of the canopy where average light intensities were lower than 150 µmol m^{-2} s^{-1}. The light conditions in the greenhouse during this time of the year may have only minor effects on ASC levels. It is only during over-ripening from red to extreme red that ASC increased which is in line with other studies (Ioannidi et al., 2009; Yahia et al., 2001). This response is not related to irradiance. The limited increase of ASC with prolonged development is attributed to the activation of the galacturonate pathway (Agius et al., 2003). The galacturonate pathway converts carbon released from cell wall breakdown to ASC. Its contribution in red ripe tomato fruit is limited while the pathway is presumably inactive in green fruit (Badejo et al., 2012). As in the current experiment there was an increase of approximately 20%, it is hypothesized that this is the result of both the primary (D-Man/L-Gal) and galacturonate pathways.

4.2 Light improves ASC levels of tomato fruit by local biosynthesis

Broadband visible light can increase ASC levels in several species. This effect has been proven for both leaves (Bartoli et al., 2006; Fukunaga et al., 2010; Massot et al., 2012) and fruit (Labrie and Verkerke, 2012; Li et al., 2010, 2009; Ntagkas et al., 2016). In line with previous work, ASC levels are higher in fruit kept in light compared to darkness. Leaf irradiation may also regulate ASC in fruit as ASC is synthesized in mature leaves and transported through phloem sieve tube elements to the fruit (Hancock et al., 2003). However, regulation of ASC in tomato fruit is more dependent on fruit irradiance compared to leaf irradiance (Gautier et al., 2008). In the current work it has been shown that fruit irradiation increases ASC levels by up to approximately 500%. Translocation of ASC from the leaves to the fruit was excluded as the fruit were placed in light after detachment from the plant and removal of the calyx. This indicates that increase of ASC by fruit illumination is due to light effects on biosynthesis, recycling and/or turnover locally in the fruit. Various genotypes of different fruit sizes, ranging from 20g to 160g average fruit weight, respond similarly. This suggests a universal effect of light on ASC in tomato fruit.

Tomato fruit from all experiments have been harvested from the same greenhouse compartment at different seasons of the year. Fruit for Exp. 2 have been harvested in spring while fruit for all other experiments have been harvested in summer. The temporal variation of irradiance (higher daily quantum integral in summer compared to spring) is the reason for the difference in starting levels (t=0) of ASC between Exp. 2 and the rest of the experiments.

In experiment 3, the maximum ASC levels after 7 days were achieved at 300 µmol m^{-2} s^{-1} (about 35 mg/100g of fresh weight). In experiment 2, ASC levels after 7 days exposure to 500 µmol m^{-2} s^{-1} were slightly higher (about 40 mg/100g of fresh weight). In experiment 3, ASC levels at 600 µmol m^{-2} s^{-1} were lower than 300 µmol m^{-2} s^{-1}. This indicates that at a 7-day treatment the optimal irradiance for stimulating ASC in tomato fruit is around 500 µmol m^{-2} s^{-1}, with irradiances above 600 µmol m^{-2} s^{-1} being supra-optimal. A possible explanation

for this reduction is that ASC may be utilized in encountering the high oxidative load observed at high irradiances.

Specific spectra of the visible part of the spectrum have an effect on ASC in green tissue. *Phaseolus vulgaris* leaves grown under low red:far-red had lower ASC levels compared to higher red:far-red (Bartoli et al., 2009). In accordance, ASC was higher in tomato fruit kept in additional red light than when kept under additional far-red with the same irradiance. This might be associated with either signalling through phytochrome or the extra photosynthetically active radiation of the red-light treatment. ASC increased also in leaves under blue light (Lester, 2006; Ohashi-Kaneko et al., 2007). The results of the current work are in line with this, as ASC increased (146%) when fruit were kept in broadband light with additional blue. When broadband light was compared to light with high proportion of either red or green, no additional effect of the latter was observed. In all light treatments, the white background light was supplied at 100 µmol m^{-2} s^{-1} which is not expected to increase ASC. Signal limitation of ASC increase due to lack of specific spectra is also not expected. It can be concluded that spectral effects are limited that cryptochromes and phototropins do not play an important role. ASC levels in the supplementary far-red treatment were higher than initial levels and fruit stored in darkness. This is not likely explained by the white background light, nor the additional PAR form the far-red lamps (5 µmol m^{-2} s^{-1}) and might be a phytochrome related effect. Ascorbate peroxidase synthesis has been proposed to be regulated by phytochrome (Thomsen et al., 1992).

4.3 Light increases ASC levels in harvested tomato fruit via effects on photosynthesis

ASC in irradiated fruit ceased to increase when the fruit lost their green colour by entering the breaker stage. Similarly, red fruit did not increase in ASC in response to light, whereas green fruit achieved higher ASC levels when kept in more than 150 µmol m^{-2} s^{-1}. This suggests that photosynthetic activity is essential for ASC accumulation. The involvement of photosynthesis in light regulation of ASC is also supported by the spectral treatments. Light spectra that are expected to result in higher photosynthetic rates (white, blue and red) resulted in higher ASC level in tomato fruit compared to darkness and spectra that result in lower photosynthesis (far-red). No considerable differences in photosynthesis are expected between the red, blue and green light treatments (Paradiso et al., 2011) which is in line with no differences observed in ASC between these treatments. Green light has beneficial effects on ASC potentially due to light absorption from the tissues below the pericarp as it penetrates deeper in the fruit. Expected photosynthetic rates for the spectral treatments correlate with ASC levels for all spectral treatments. It can be concluded that a minimum amount of chlorophyll in the tissue of tomato fruit is essential for light regulation of ASC.

Involvement of photosynthesis in light regulation of ASC has been previously proven in leaves but not in fruit. In leaves, high irradiances result in higher photosynthetic electron transport. The latter regulates the plastoquinone redox state which affects the gene expression of ASC related enzymes (Karpinski et al., 1997). Arabidopsis leaves treated with ATZ and DCMU (photosynthetic inhibitors) did not achieve higher ASC levels when placed under light, compared to non-treated plants. This is attributed to reduced activity of the D-Man/L-Gal pathway (Yabuta et al., 2007). In the time course experiment, photosynthetic rates were higher when fruit kept in light compared to darkness (Fig. 3A). Inhibition of

photosynthesis with DCMU also reduced ASC levels in mature green tomato fruit but not in red fruit (Badejo et al., 2012). It can be concluded that a minimum photosynthetic rate in tomato fruit is essential for light induced increase of ASC.

Respiration is also related to ASC. The last enzyme of the main biosynthetic pathway (GLDH) is located in mitochondria (Bartoli et al., 2000). GLDH is part of complex I of the respiratory electron transport chain (Schimmeyer et al., 2016). CytC is an electron acceptor from GLDH (Leferink et al., 2008). When cytC is oxidized, the enzymatic activity of GLDH increases resulting in ASC accumulation, given that the substrate for this reaction is sufficient (Bartoli et al., 2000). In the time course experiment, respiratory rates and ASC levels in different light treatments did not correlate. Respiratory CO_2 emissions were lower in fruit at 500 µmol m^{-2} s^{-1} compared to darkness at day 2. Respiration rates were similar in dark and light treated fruit at the end of the treatment. Light suppression of dark respiration in darkness might be a potential explanation of such a response (Sharp et al., 1984). The involvement of respiration in light regulation of ASC in tomato fruit cannot be dismissed in its entirety. Treatment of arabidopsis leaves with respiratory inhibitors (KCN and rotenone) proved that respiration is essential for the achievement of maximal ASC biosynthetic rates (Bartoli et al., 2006; Millar et al., 2003).

5. Conclusions

ASC levels increased in detached mature green fruit when they were exposed to higher irradiances. ASC levels of red tomato fruit did not respond to irradiance treatments. Spectral effects on ASC were limited. Furthermore, the ASC levels increase when the fruit still contain considerable amounts of chlorophyll and is manifested across a range of different sized cultivars. Therefore, it can be concluded that the light signal for increase of ASC is perceived only by chlorophyll containing fruit and fruit illumination is sufficient for considerable ASC upregulation in the fruit pericarp. This effect is mostly independent of the light spectrum. The rate of fruit photosynthesis correlated with the light induced increase in ASC. There was no correlation between the respiratory rate of the fruit and ASC levels. Therefore, it can be concluded that the positive effect of light on ASC levels of tomato fruit is mediated primarily through fruit photosynthesis.

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